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Allison, Gordon G.; Hughes, John-Wayne; Morris, Phillip; Robbins, Mark P.

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Compounds and genes for enhanced protein assimilation and digestibility in forage legumes: Altering condensed tannins content in the leaves of forage legumes

G. Allison, J.W. Hughes, P. Morris and M.P. Robbins
Institute for Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion, SY23 3EB, UK

SUMMARY – A large proportion of the protein present in alfalfa is lost in the rumen as a consequence of protolysis and deamination. This consequentially decreases animal productivity and in some instances causes pasture bloat, a chronic digestive disorder that is endemic to cattle. However, *in vitro* and *in vivo* studies of alternative forage species have shown that the presence of low levels (3-4%) of condensed tannins (CT, or proanthocyanidins) increase animal productivity and lessens the likelihood of pasture bloat. One aim of forage legume breeders is therefore to induce the accumulation of desirable levels of CT biosynthesis in CT negative forages such as white clover and alfalfa. However, interspecific crosses and somatic hybridisation have not achieved this goal. The European Union CAGED Project (Compounds And GEnes for enhanced protein assimilation and Digestibility of forage legumes-FAIR PL 98 4068, http://caged.irmgpf.pg.cnr.it/) takes a different approach to attain this goal. The aim of the project is to elucidate the biosynthetic pathway of CT synthesis by isolating and functionally assessing key genes from the CT pathway. This knowledge will guide our assessment of the options for modifying commercially grown forage legumes to express desirable levels of CT by use of modern breeding methodologies, gene-targeting and mutation. Central to our strategies is the CT accumulating species *Lotus corniculatus*. This model crop is a *bone fide* forage legume that is polymorphic for CT and is well adapted to *in vitro* manipulation and genetic transformation. We at IGER are collaborating with our colleagues at IRMGPF to isolate CT genes from *L. corniculatus*, characterising their regulation and evaluating their precise role in CT synthesis. In addition, we are closely involved with the Genetics and Breeding of Forage Legumes Group at INRA, who have extensive experience of forage digestibility and protein degradation analysis.

Key words: Condensed tannins, forage quality, *Lotus corniculatus*, protein assimilation, CAGED.

RESUME – *"Composés et gènes pour une augmentation de l'assimilation des protéines et de la digestibilité chez les légumineuses fourragères: Modification de la teneur en tannins condensés dans les feuilles des légumineuses fourragères". Une partie importante des protéines présentes dans la luzerne n'est pas assimilée au niveau du rumen, dû à des phénomènes de protéolyse et de désamination. Cette perte entraîne une diminution de la productivité animale, et peut même, dans certains cas, être à l'origine de météorisation, une maladie digestive chronique et endémique chez les bovins caractérisée par un gonflement de l'estomac. Cependant, des études* in vitro *et* in vivo *sur d'autres espèces fourragères ont montré que la présence en faible quantité (3-4%) de tannins condensés (TC, ou proanthocyanidines) améliore la productivité animale et réduit les risques de météorisation. Par conséquent, il est intéressant pour les sélectionneurs de légumineuses fourragères d'induire l'accumulation de biosynthèse de TC a des niveaux désirables chez les fourragères non-accumulatrices de TC telles que le trèfle blanc et la luzerne. Les croisements interspécifiques et l'hybridation somatique ayant échoué, le projet CAGED (Compounds And GEnes for enhanced protein assimilation and Digestibility of forage legumes-FAIR PL 98 4068, http://caged.irmgpf.pg.cnr.it/) de l'Union Européenne a pris une approche différente à ce problème. Le but est d'élucider les voies métaboliques de la synthèse des TC en isolant les gènes impliqués et en testant leurs fonctions. Les connaissances ainsi acquises nous aideront à évaluer les options pour faire exprimer des niveaux désirables de TC aux variétés commerciales de légumineuses fourragères, par l'utilisation de méthodes modernes de sélection, de ciblage génétique et de mutation.* Lotus corniculatus*, espèce accumulatrice de TC, se trouve au centre de nos stratégies. Légumineuse fourragère polymorphique pour les TC, cette espèce modèle est bien adaptée aux manipulations* in vitro *et à la transformation génétique. À l'IGER, nous collaborons avec les chercheurs de l'IRMGPF pour isoler les gènes impliqués dans la biosynthèse des TC chez* Lotus corniculatus*, mais aussi pour caractériser leur régulation et définir leur rôle précis dans la synthèse des TC. De plus, nous travaillons en collaboration avec la Station d'Amélioration des Plantes Fourragères de l'INRA de Lusignan, qui est spécialisée dans l'étude de la digestibilité des fourrages et l'analyse de dégradation protéique.*

Mots-clés : *Tannins condensés, qualité du fourrage,* Lotus corniculatus*, assimilation des protéines, météorisation au pâturage, CAGED.*

Introduction

Alfalfa (*Medicago sativa*) is a forage legume that is widely cultivated in Northern Europe and is the most important perennial forage crop grown in the USA (Jung *et al*., 1997). *In vitro* (Broderick and Albrecht 1997) and *in sacco* studies (Julier *et al.*, this volume) of alfalfa digestibility have shown that up to one quarter of ingested alfalfa protein is degraded in the rumen within 2 to 8 hours. This breakdown of plant protein is effected by microbial proteolysis and deamination, and by the action of proteases that are present in the forage (Theodorou *et al.*, 1999). These processes result in nitrogen being lost from the rumen, which consequently lowers animal productivity (Cohen, 2001). In addition, rapid degradation of dietary protein can result in pasture bloat, a chronic condition that is endemic to cattle (Berg *et al.*, 2000). Protein in alfalfa leaves is more susceptible to degradation in the rumen than that present in stems. This is due to the larger amounts of lignin and fibre present in the stems and the decreased levels of digestible dry matter (Kuehn *et al.*, 1999). Indeed, efforts to breed bloat-safe alfalfa have so far produced varieties that have thicker cell-walls (Goplen *et al.*, 1993).

Condensed tannins (CT), or proanthocyanidins, which occur in a restricted range of forages, have the ability to interact with proteins and enzymes and can influence the digestion of plant protein in the rumen (McNabb and Waghorn, 1996). Low levels of CT (between 3% to 4%) in forage has been shown to increase animal productivity (Wang *et al.*, 1996; Adu *et al.*, 1998; Aerts *et al.*, 1999; Barry and McNabb, 1999; Woodward *et al.*, 1999). This is effected by a reduction of ruminal nitrogen loss by degradation (Reed, 1995; McNabb *et al*., 1996; Broderick and Albrecht, 1997; Robbins *et al.*, 1999; Theodorou and Barahona, 1999). In addition, there is a lowering of the incidence of pasture bloat (McMahon *et al.*, 1999; Robbins *et al.*, 1999). CT accumulation in alfalfa foliar tissues is therefore a long-standing goal of plant breeding, but one that has not yet been achieved by conventional plant breeding methods.

Alfalfa does indeed accumulate CT in its seed coat. This CT contains only trace amounts of prodelphinidin but analysis of its binding to bovine serum albumin shows it to be indistinguishable from CT isolated from the leaves of sainfoin, a highly tanniferous forage legume (Koupaiabyazani *et al.*, 1993). It is clear therefore that alfalfa already has some if not all of the enzymatic machinery necessary for CT accumulation and this could be achieved by altering the location of biosynthesis and storage from the seed coat to the leaves and stems.

Results and discussion

Sn is a member of the R family of *myc* regulatory genes from maize (*Zea mays*), where it regulates the cell specific deposition of anthocyanins in seedlings and adult cells (Tonelli *et al*., 1991). Transgenic lines of *L. corniculatus* var. Leo isogenic genotype S50 transformed with the constitutively expressed *Sn bol3* allele are altered in their accumulation of CT accumulation in root and foliar tissues (Damiani *et al*., 1999). In many lines there is a marked increase in CT accumulation relative to control plants. This can be seen clearly in Fig. 1, which shows leaves from an up-regulated transgenic line and a control line in which the CT containing cells have been stained purple with DMACA (Li *et al*., 1996). This effect of the transgene is specific to CT and anthocyanin biosynthesis and analysis of other key flavonoid end-products in leaf and stem samples shows no alteration in the levels of lignin and flavones (Fig. 2).

Fig. 1. Leaves of *L. corniculatus* lines stained for the presence of condensed tannins with DMACA: (A) untransformed control line, (B) *Sn* transgenic line *Sn* 11.

IGER and colleagues in CNR and INRA are united by the European Union CAGED project (Compounds And GEnes for enhanced protein assimilation and Digestibility of forage legumes-FAIR PL 98 4068, http://caged.irmgpf.pg.cnr.it/) in their investigations of CT biosynthesis and its qualitative and quantitative effects on protein assimilation in the rumen. We are interacting with IRMGPF to isolate genes that are involved in the biosynthesis and regulation of CT in *L. corniculatus* by subtractive screening methodologies using CT up-regulated *Sn* transgenic and control lines. We have also used sequence data from public domain data bases to design oligonucleotide primers, which we have used to isolate the majority of the flavonoid pathway genes by PCR. These sequences are being functionally assessed at IGER by analysis of their effects on the pattern of CT accumulation when silenced in *L. corniculatus*. We are also assisting the Genetics and Breeding of Forage Legumes Group at INRA to characterise the effects of CT abundance and monomer composition on ruminal protein degradation by use of *in sacco* and *in vitro* methods. Our contribution has been to produce quantities of purified CT from a number of tropical and non-tropical forage plants, which will be used to characterise the effects of CT monomer composition. In addition, we have prepared 11 separated leaf and samples of *Lotus* species that have been used to assess the effect of CT content on dry matter digestibility and protein degradation (Julier *et al*., this volume).

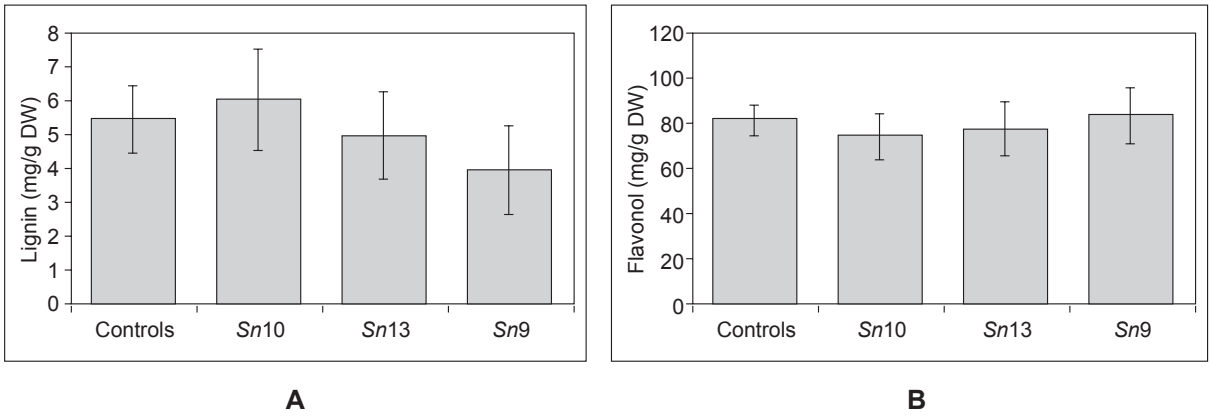

Fig. 2. Analysis of lignin (A) and flavonol (B) in control and *Sn* transgenic lines of *L. corniculatus.*

We anticipate that the knowledge gained in the CAGED project of CT biosynthesis, regulation and effects on forage digestibility will be invaluable in identifying candidate genes for modifying the pattern of CT biosynthesis in alfalfa by technologies including chaemeroplasty and gene targeting, mutation and other modern plant breeding methods.

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